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Penetration and Infection Processes of *Alternaria brassicicola* on Cauliflower Leaf and *Alternaria brassicae* on Mustard Leaf: A Histopathological Study

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Abstract: *Alternaria* blight caused by *Alternaria brassicicola* (Schwein) Wiltshire and *Alternaria brassicae* (Berk) Sacc. is one of the most serious diseases of Cauliflower [*Brassica oleracea* (L.) var. *botrytis*] and Mustard [*Brassica juncea* L.] grown as edible vegetable and oilseed crop, respectively in India. As resistance against these pathogens is not found till now therefore, the exploration of the host-pathogen interaction is much needed. In the present study, the cauliflower-*A. brassicicola* and mustard-*A. brassicae* interactions were studied at microscopic level using light and electron microscope. The initial infection processes involve conidia germination, penetration and colonization on the plant surfaces. Several germ-tubes developed from conidia and colonize extensively across the leaf surfaces. Penetration in the plant surface, either directly through the epidermis or via stomata occurred with occasional appressoria formation. Least aggressive *A. brassicae* isolate was found to infect mustard leaf by spreading over epidermal cells only. Finally, hyphae colonize the leaf cells forming network collapsing epidermal cells to form necrotic lesions which may be due to release of toxin.

Key words: *Alternaria brassicicola*, cauliflower, *Alternaria brassicae*, mustard, light microscopy, SEM

INTRODUCTION

Leaf spot is a common term applied to a disease that normally affects the foliage of annual, perennial crops and forest trees, the majority of which are caused by a variety of fungal pathogens and also by bacteria. Among various leaf spot fungal pathogens, *Alternaria* species is one of the major production constraints in most of the field and horticultural crops. In vegetable Brassica seeds, especially white cabbage and cauliflower [*Brassica oleracea* (L.) var. *botrytis*], *Alternaria brassicicola* (Schwein) Wiltshire (Maude and Humpherson-Jones, 1980; Humpherson-Jones, 1985; Maude *et al.*, 1984; Kubota *et al.*, 2006; Deep and Sharma, 2012; Sharma *et al.*, 2013a) is the dominant pathogen whereas in oilseed rape, especially mustard [*Brassica juncea* (L.)], *A. brassicae* (Berk) Sacc. is dominant (Sharma *et al.*, 2013b). These species can survive in seeds for several months at different temperatures and relative humidity (Kumar and Gupta, 1994; Abul-Fazal *et al.*, 1994) and the disease is spread during the growing season having cool and moist weather by wind-blown or rain-splashed spores (Rotem, 1994; MacKinnon *et al.*, 1999; Oliver *et al.*, 2001). The pathogen attacks most parts of the plant like seed, stem, leaf,

inflorescence and fruits and it is thought to induce its chlorotic effect by release of phytotoxins (Jung *et al.*, 2002). *Alternaria* leaf spots usually appear on the oldest leaves first and later spread to the newer leaves towards the tips (Chattopadhyay, 1999; Meena *et al.*, 2010; Deep and Sharma, 2012). Leaf spots appear as bright to pale yellow or tan flecks on the upper leaf surface which may be surrounded by light green or yellow halos. The older spots are somewhat circular to irregularly lobed and are light brown-black in colour. These old spots may or may not have characteristic concentric rings. Lesions on the petioles and stems are dark brown and often coalesce and girdle the stems. The leaf spots increase in size and number and coalesce and cause leaf blights.

The disease cycle is simple as no teleomorph found till now (Aveling *et al.*, 1994). Conidia are produced on leaf surface and dispersed by wind. Conidia germinate in availing suitable condition getting moisture and produce toxin before penetrating host tissue. Penetration on the leaf surface can occur directly or through stomata (Dehpour *et al.*, 2007). To study the penetration and infection process of the *A. brassicicola* and *A. brassicae* on economically important cauliflower and mustard leaves, respectively, the present study has been carried out.

MATERIALS AND METHODS

Inoculum preparation: Seven day old *Alternaria brassicicola* (CaAbcT3 isolate), *A. brassicae* (CaAbD5, highly aggressive and CaAbR3, least aggressive isolates) grown on Potato Dextrose Agar (PDA) plates at 25°C were flooded with distilled water and spores were released by agitation with a sterile brush. The resulting spore suspension density was adjusted to 4×10^4 spores mL⁻¹ by haemocytometer. One drop of Tween-20 1 mL⁻¹ suspension was added as a wetting agent.

Plant material: The cauliflower cultivar namely, Pusa Deepali (early-maturing) and mustard cultivar namely, Pusa Jagganath seeds were obtained from Division of Vegetable Sciences and Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi, respectively. The cauliflower seeds were sown in nursery beds during October, 2011-12 seasons. Seedlings were transplanted after two weeks in fields with a spacing of 30×40 cm. Similarly mustard seeds were also sown in field. 45-60 days old leaves were taken for the study further.

Detached leaf inoculation: Leaves were properly washed under running tap water and then surface wiped off with 70% alcohol and 2 µL of 4×10^4 spores mL⁻¹ spores were inoculated with a fine needle (Dispovan, 2.5 mL) while sterile distilled water was applied on control (Sharma *et al.*, 2004). The leaves were placed inside moist chambers in green house conditions and were observed for appearance of disease symptoms on 3rd day after inoculation. Development of disease symptoms was observed on third day after inoculation in the inoculated leaves while controls remained free from symptoms.

Light microscopy: To observe the infection process of *Alternaria brassicicola* and *A. brassicae* on leaves of cauliflower and mustard, infected and uninfected leaf samples were properly washed with distilled water twice and then dried. Thin section (1") crosswise cut was cut into transparent slices with the help of a sterilized blade or scalpel. Thin cross sections of leaf samples were decolorized with 0.15% (w/v) TCA in Ethanol: Chloroform (3:1) then wet mounted with a drop of 0.01% lactophenol cotton blue on a glass slide and covered with a cover slip. The slides were observed under light microscope (Zeiss AX10Star Plus) at 50-60 Hz and 65 V. More than fifty samples were examined to confirm the results.

Scanning electron microscopy: Samples of leaf pieces (size: ~2 mm) after infection collected were washed in

0.1 M phosphate buffer three times at an interval of 15 min at a temperature of 4°C. Samples were post fixed in 1% Osmium tetroxide in the same buffer for 2 h at 4°C and again washed in 0.1 M phosphate buffer three times as above. Dehydration with different concentrations of acetone (30, 50, 70, 80, 90, 95% once each and 100% twice) was carried out at 15 min interval at 4°C. Critical point drying (optional) with liquid CO₂ (31.5°C at 100 PSI) was done. The dried materials were then adhered on to the aluminium specimen mounts with colloidal silver paste and then sputter coated (Emitech CA7625 Carbon Accessory) with gold palladium (~24 nm thickness). The specimens were examined and photographed on a scanning electron microscope (Zeiss EVOMA10) at 20 kV/EHT and 10 Pa between 2-100 X zoom. At least 30 leaf disks were observed to verify the result.

All the three isolates of *A. brassicicola* (CaAbcT3) and *A. brassicae* (CaAbD5 and CaAbR3) grown on PDA, were also observed under light microscope and scanning electron microscope as above to observe the morphology microscopically.

RESULTS AND DISCUSSION

Conidia of *Alternaria brassicicola* isolate CaAbcT3 was found to be small and septate ranging from 7-35 µm long with short beak (Fig. 1). Chain of conidia were found (Fig. 1b) having 2-8 conidia forming germ tubes which were branched infrequently with average size of 8-200 µm in length. Similarly conidia of *A. brassicae* CaAbD5 (Fig. 1c) and CaAbR3 (Fig. 1d) were also found to be small and septate having 3-5 septa and 10-45 µm long with long beak. This was morphologically similar to leaf spot causing pathogen *A. brassicae* in vegetable and oil seed crops (Sharma *et al.*, 2013c). As a dominant pathogen in cauliflower (Deep and Sharma, 2012; Sharma *et al.*, 2013a), *A. brassicicola* infected cauliflower leaf tissue revealed damage to cell and cell wall as compared to the healthy tissue. The damaged portions were found to be discoloured (Fig. 2a) as a result of interaction with the fungus. It may be due to production of lytic enzymes like polygalacturonase, pectin lyase, pectin methylesterase, cellulase or fungal toxins either Host Specific Toxin (HST) or Non-Host Specific Toxin (NHST) (Nozaki *et al.*, 1997; Berto *et al.*, 1997; Gautam *et al.*, 2012; Jahangeer *et al.*, 2005). In some infected cell, deposition of lignin to the cell wall was found (Ramm, 1962). *A. brassicicola* conidia both germinated and ungerminated were adhered strongly to the leaf surface. Light microscopy results showed profusely growing germ tubes from the conidia dispersed throughout the infected leaf surface lesion (Fig. 2b). Germ tube was also found to penetrate the leaf cell



Fig. 1(a-d): Light micrograph (40X) of conidia and mycelia formed by *Alternaria brassicicola* and *Alternaria brassicae* (a) Mature small beaked conidia of *A. brassicicola* with multiple septa, (b) *A. brassicicola* conidia arranged in chain form, (c) Mature conidia of *A. brassicae* isolate; CaAbD5 and (d) *A. brassicae* isolate; CaAbR3 with multiple septa and long beak

through stomata (Fig. 2c, d) forming appresoria. The formation of several germ tubes and appresoria from a conidium is a common feature of this genus, as reported in *A. porri* (Giri *et al.*, 2013; Aveling *et al.*, 1994; Everts and Lacy, 1996), *A. linicola* (Vloutoglou *et al.*, 1996), *A. alternata* (Gupta *et al.*, 1997) and *A. cassia* (Mims *et al.*, 1997).

Scanning electron microscopy also resulted into similar observations as that of light microscopy. Healthy cauliflower leaf (Fig. 3a) was found to have normal epidermal cells without any damage while *A. brassicicola* (CaAbcT3) infected leaf surface (Fig. 3b) was spreaded over by the hypahal growth intercellularly entering with or without forming appresoria near and through the stomatal opening. Extensive growth of germ tubes formed a hyphal network on the infected leaf tissue (Fig. 3c). Some hyphal structures of *A. brassicae* (highly

aggressive; CaAbD5) were found to spread around the intercellular space of the mesophyll and parenchyma tissue through the wounds of the mustard leaf (Fig. 3d). Most of the *A. brassicicola* conidia and germ tube penetrate inside the epidermal cell directly through stomata while other passed near the stomata without forming appresoria (Fig. 4a, b). Similarly young conidia of *A. brassicae* were found to spread over the mustard leaf tissue (Fig. 4c) and movement of germtube was found through epidermal cells of mustard leaf not through stomata (Fig. 4d).

A distinct difference between highly aggressive and least aggressive isolate of *A. brassicae* was found over the infection process on mustard leaf. The highly aggressive isolate of *A. brassicae* (CaAbD5) was found to invade through stomatal opening of the host mustard leaf (Fig. 5a) as that of *A. brassicicola* infection on cauliflower

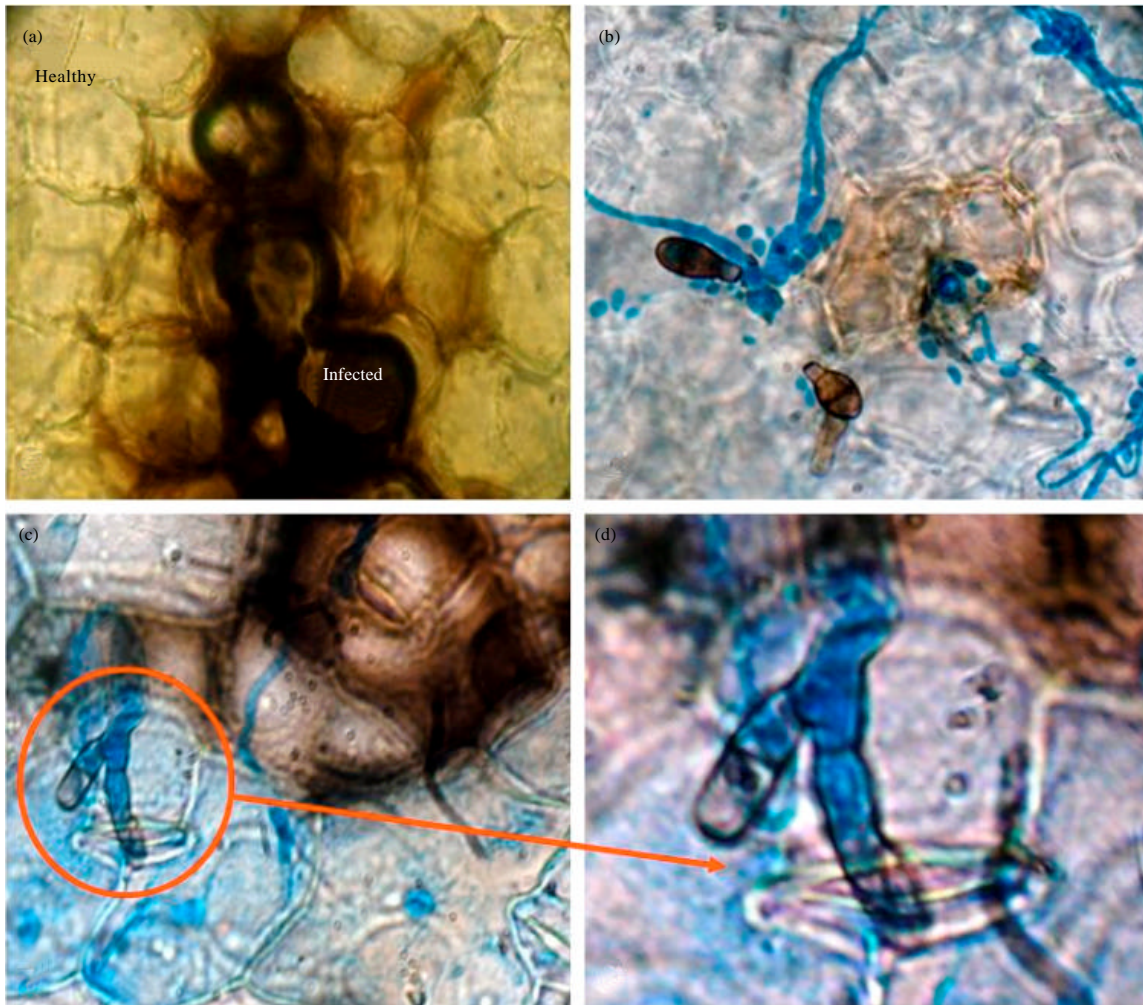


Fig. 2(a-d): Light micrograph of cauliflower leaf infected with *Alternaria brassicicola* (40X) (a) Cauliflower leaf tissue showing difference between healthy and infected portion due to *Alternaria* toxin, (b) *A. brassicicola* conidia and mycelia spread on cauliflower leaf cells and (c, d) Germtube of *A. brassicicola* penetrating the cauliflower leaf tissue through stomata opening

leaf while the least aggressive isolate of *A. brassicicola* (CaAbR3) was found to infect the mustard leaf by spreading its hyphae over the epidermal cells moving adjacent to stomata (Fig. 5b).

The microscopic infection process of *A. brassicicola* on cauliflower and *A. brassicicola* on mustard observed in the present study was similar to that of infection by wild type *A. brassicicola* on cabbage leaf surface where appressorium was developed from the conidia to penetrate plant surface through stomata and wounds to colonize its hyphae (Scott, 2008). Another microscopic observations also revealed that the fungus, *A. brassicicola* penetrate *Arabidopsis thaliana* siliques through cellular junctions,

replum and stomata and into seed coats either directly or through cracks (Pochon *et al.*, 2012). Giri *et al.* (2013) also found similar mode of infection and penetration by *A. brassicicola* on mustard leaf tissue. Similar results were found by some other *Alternaria species* on a range of hosts (Dehpour *et al.*, 2007; Isshiki *et al.*, 2001; Kiely, 1964; Mims *et al.*, 1997; Pegg, 1966; Ruehle, 1937; Saad and Hagedorn, 1969). Van Dyke and Trigino (1987) reported that the cells in the substomatal area beneath appressoria were necrotic with no evidence of fungal invasion in the tissue. Hyphal penetration was seldom observed prior to necrosis of mesophyll cells and that the death of these cells in advance of fungal penetration the

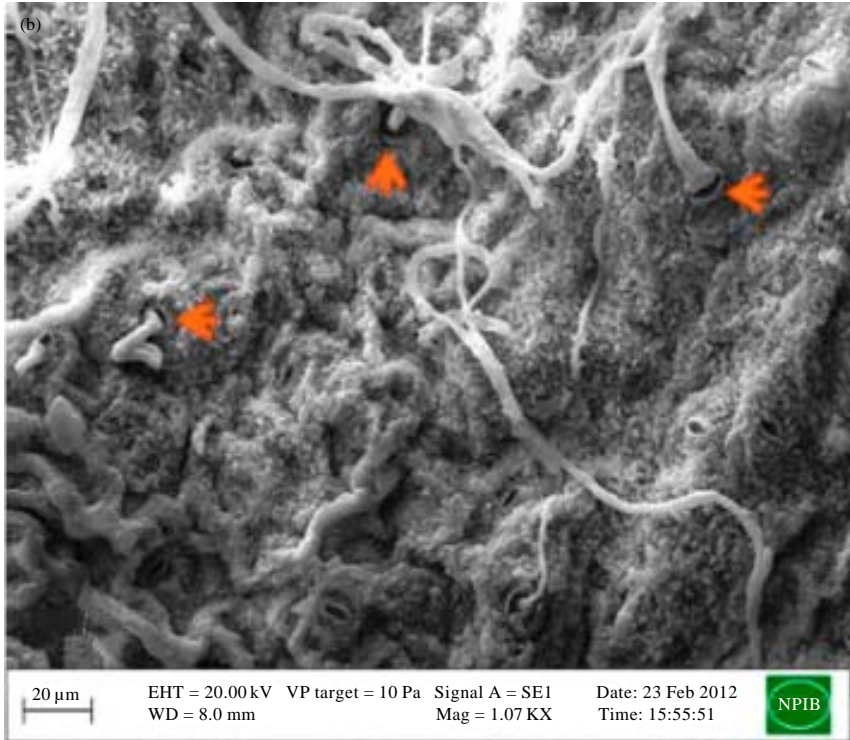
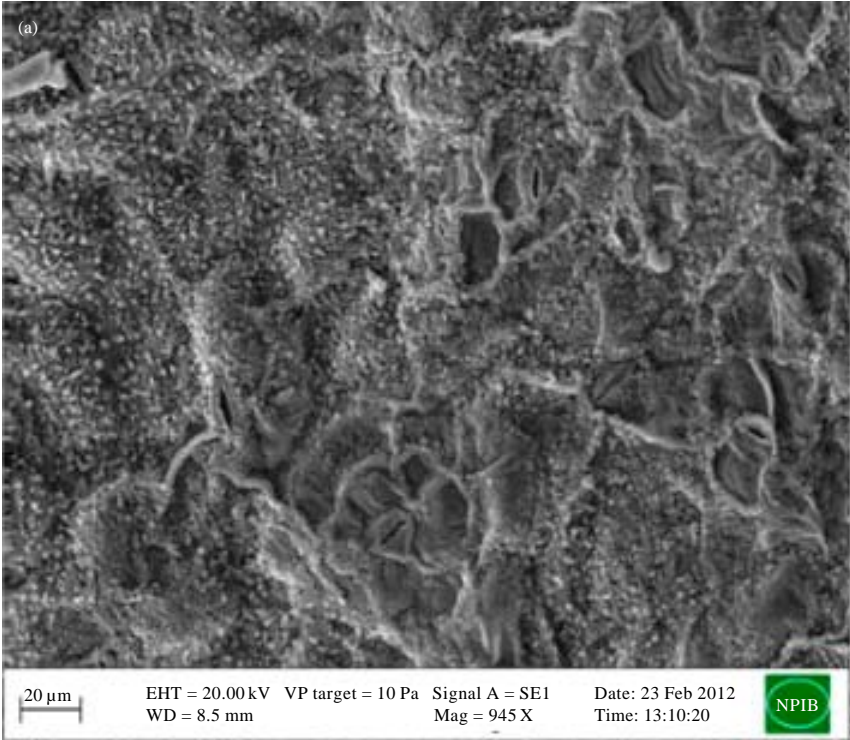


Fig. 3(a-d): Continue

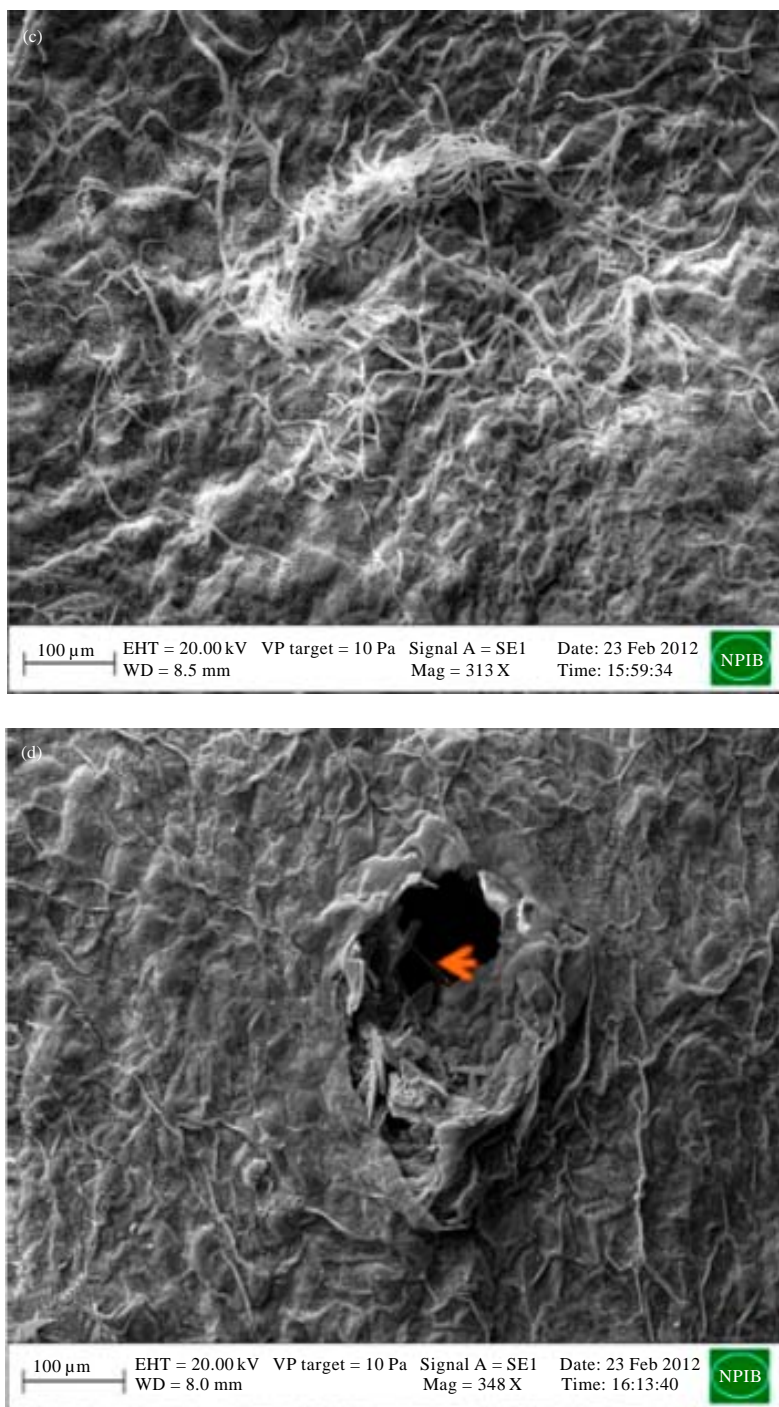


Fig. 3(a-d): Scanning electron micrograph of cauliflower and mustard leaves infected with *Alternaria brassicicola* and *Alternaria brassicicola* (a) Healthy cauliflower leaf (Bar = 20 μ m). (b) Infected cauliflower leaf with movement of *A. brassicicola* mycelia through stomatal opening, (c) Spreading of hyphal network of *A. brassicicola* (CaAbcT3) on infected cauliflower leaf surface and (d) *A. brassicicola* (Highly aggressive; CaAbd5) hyphae growing intercellularly passing through damaged epidermal cells of the necrotic lesion

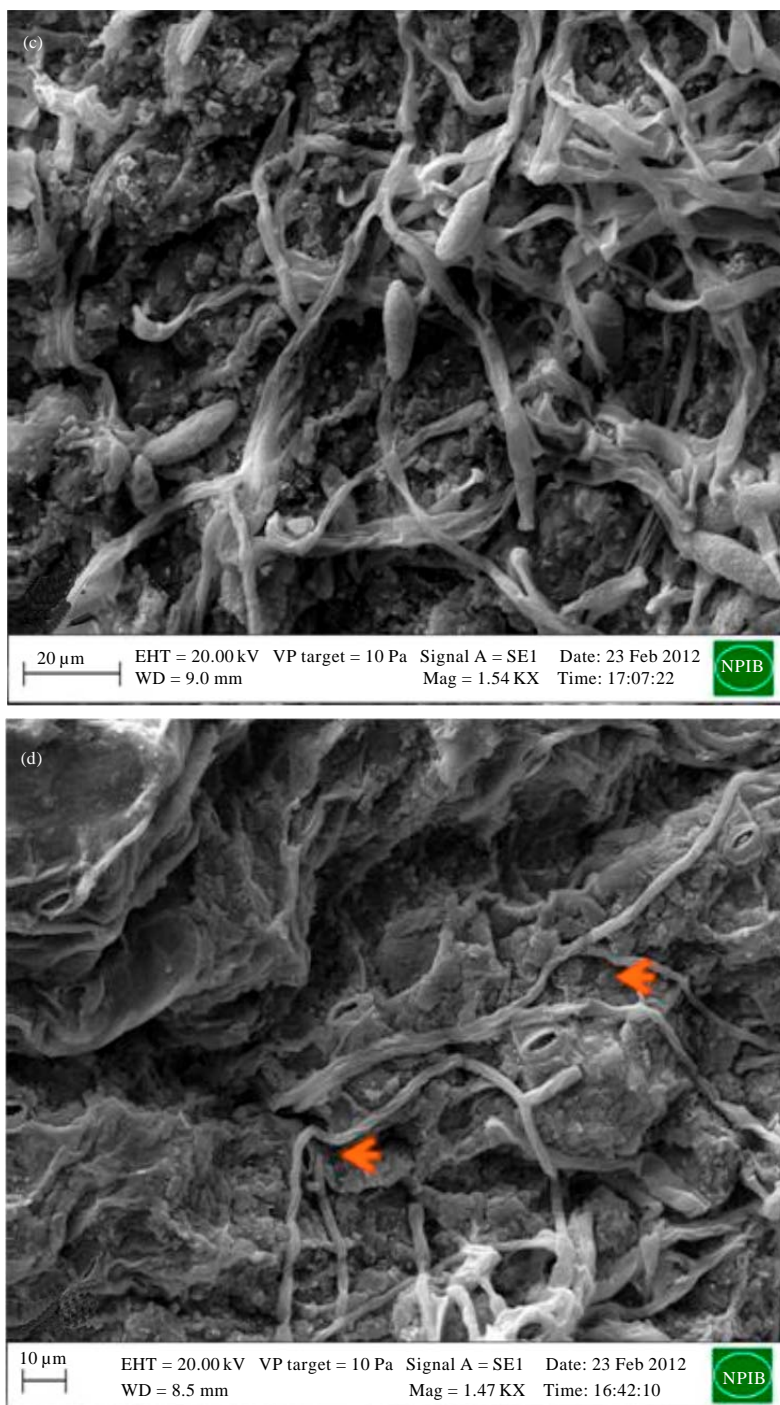


Fig. 4(a-d): Scanning electron micrograph of penetration of conidia and germtube of *Alternaria brassicicola* and *Alternaria brassicae* on cauliflower and mustard leaf, respectively, (a, b) Direct penetration of *A. brassicicola* (CaAbcT3) hyphae through the epidermis and stomata (Bar = 20 μm), (c) Spreading of *A. brassicae* (CaAbD5) hyphae and conidia over infected mustard leaf (Bar = 20 μm) and (d) Movement of *A. brassicae* (CaAbD5) hyphae and germtube through epidermal cells of mustard leaf not through stomata (Bar = 10 μm)

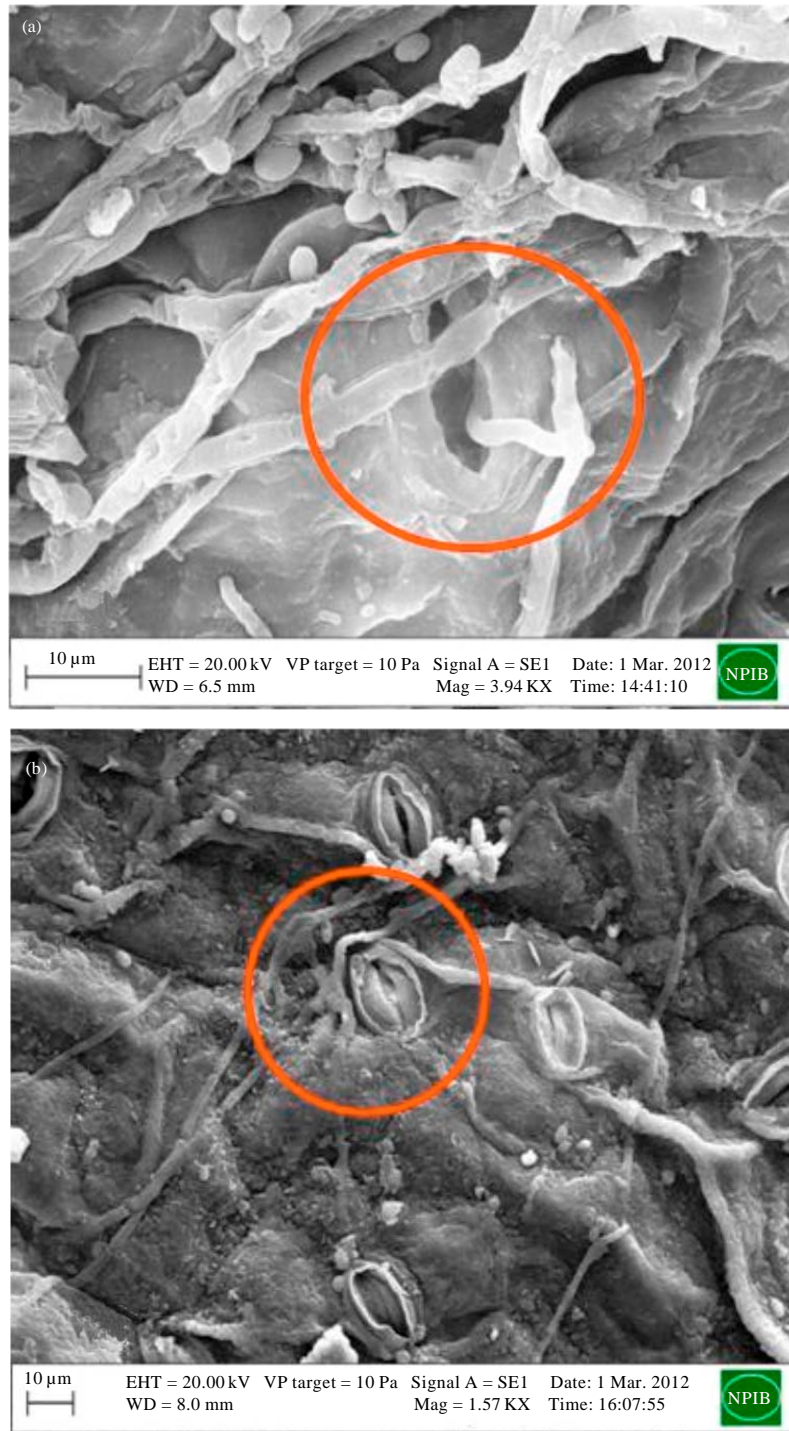


Fig. 5(a-b): Scanning electron micrograph of movement of germtube and hyphae of *Alternaria brassicae* on mustard (Pusa Jagganath) leaf (a) Penetration of germtube of *A. brassicae* (highly aggressive; CaAbD5) through stomatal opening and (b) Spreading of *A. brassicae* (least aggressive; CaAbR3) hyphae over the epidermal cells of leaf, passing near the stomata (Bar = 10 µm)

action of diffusible toxins. Dehpour *et al.* (2007) found that secondary hyphae developed from primary hyphae and grew in the intercellular spaces and also penetrated and grew intercellularly with in the epidermis.

Both *A. brassicicola* and *A. brassicae* enter the leaf tissue of *B. oleracea* var. *botrytis* and *B. juncea*, respectively both by direct (epidermal) and stomatal penetration which was confirmed by same penetrations process in *A. ricini* on castor leaf and *A. porri* on onion leaf (McKenzie *et al.*, 1993; Aveling *et al.*, 1994; Suheri and Price, 2000; Babu *et al.*, 2009). The least aggressive *A. brassicae* isolate showed somewhat deviation in the present study by moving only over epidermal cells of *B. juncea*. Similar results were found by *A. linicola* on water agar, linseed leaves (Vloutoglou *et al.*, 1996) and in *A. alternata* on mulberry leaves (Gupta *et al.*, 1997) whereas, penetration only through stomata was observed in case of *A. eichhorniae* on water hyacinth leaves (Shabana *et al.*, 1997) and in *A. alternata* on grape fruits (Swart *et al.*, 1995).

CONCLUSION

In the present study, only the process of infection and penetration of *A. brassicicola* through cauliflower leaf tissue and *A. brassicae* through mustard leaf tissue were studied and confirmed with previous studies done on interaction of other *Alternaria* spp. on wide range of hosts. Penetration and infection directly by epidermal cell and/or stomatal opening was found. Hyphae of the both the *Alternaria* spp. on both Brassica host was found to spread like net formation which lead to damage the leaf cell forming necrotic lesions. This is initial study of infection process of the important leaf spot causing pathogen in crucifer. The mechanism behind the host and pathogen interactions can be studied further.

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